

Spinal Adrenergic and Cholinergic Receptor Interactions Activated by Clonidine in Postincisional Pain

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Background: Previous pharmacologic and molecular studies suggest that the α_2 -adrenoceptor subtype A is the target for spinally administered α_2 -adrenergic agonists, *i.e.*, clonidine, for pain relief. However, intrathecally administered α_2 C antisense oligodeoxynucleotide was recently reported to decrease antinociception induced by clonidine in the rat, suggesting non-A sites may be important as well. The current study sought to determine the subtype of α_2 adrenoceptors activated by clonidine in a rodent model for human postoperative pain, and to examine its interaction with spinal cholinergic receptors.

Methods: Postoperative hypersensitivity was produced in rats by plantar incision of the hind paw and punctuate mechanical stimuli were applied around the wound 24 h after surgery. Effects of intrathecal clonidine and 2-(2,6-diethylphenylamino)-2-imidazoline (ST91) on withdrawal thresholds to the stimulus were determined. To examine the adrenoceptor subtype and its interaction with spinal cholinergic receptors, animals were intrathecally pretreated with vehicles BRL44408 (an α_2 A subtype-preferring antagonist), ARC239 (an α_2 non-A subtype-preferring antagonist), atropine (a muscarinic antagonist), and mecamylamine (a nicotinic antagonist).

Results: Intrathecal ST91 showed a significantly greater efficacy when compared with clonidine. The analgesic effect of clonidine was diminished by pretreatment with either adrenoceptor antagonist, whereas the effect of ST91 was solely blocked by ARC239 pretreatment. Atropine and mecamylamine abolished the effect of clonidine effect but not the effect of ST91.

Conclusions: Both α_2 A and α_2 non-A adrenoceptors, as well as spinal cholinergic activation, are important to the antihypersensitivity effect of clonidine after surgery. ST91 is more efficacious in this model than clonidine and relies entirely on α_2 non-A adrenoceptors.

THE α_2 -adrenoceptor agonists unquestionably yield antinociception in acute and in chronic pain states, both in animals and humans.¹⁻³ However, α_2 -adrenoceptor agonists, such as dexmedetomidine or clonidine, also produce sedation and cardiovascular depression after systematic or intrathecal injection, limiting their use to an adjuvant for analgesia. Among these compounds, 2-(2,6-diethylphenylamino)-2-imidazoline (ST91), a polar analog of clonidine, seems to be an attractive alternative to current agents since it produces analgesia in normal rats

and in rats after nerve injury without significant hypotension, bradycardia, and sedation.⁴ The favorable profile of ST91 could reflect altered distribution due to its hydrophilicity or due to receptor subtype selectivity.⁵ ST91 interacts with different α_2 -adrenoceptors than does clonidine or dexmedetomidine, as indicated by its reversal by antagonists that do not affect these agonists⁶ and by its synergy for antinociception when combined with these agonists.⁷

We have recently shown that, after peripheral nerve injury, Sprague-Dawley rats manifest remarkable changes in spinal α_2 -adrenoceptor agonist pharmacology for antinociception. First, we showed that the antinociceptive effect of clonidine in normal rats reflects activation of both α_2 A and α_2 non-A-adrenoceptor subtypes, whereas following spinal nerve ligation in animals, this effect is totally due to activation of α_2 non-A-adrenoceptor subtypes.⁸ Second, in animals with peripheral injury, but not in normal animals subjected to a noxious thermal stimulus (unpublished observations, Xavier Paqueron, MD, Research Fellow, Department of Anesthesiology, Wake Forest University School of Medicine, June, 2000), clonidine loses its antinociceptive effect after destruction or antagonism of the cholinergic receptor population when the animals were subjected to a mechanical stimulus.⁹ This indicates a major shift in α_2 -adrenoceptor mechanisms after nerve injury.

Hypersensitivity to mechanical stimuli following surgery exhibits unique pharmacology of inhibition compared with that of inflammation or nerve injury. The purpose of the current study was to evaluate the potency and efficacy of spinally administered ST91 in an experimental model for human postoperative pain when compared to clonidine. In addition, we examined which of the α_2 -adrenoceptor subtypes was activated by spinally injected clonidine and whether α_2 -adrenoceptor efficacy required interactions with spinal nicotinic and muscarinic receptors after surgery.

Materials and Methods

Surgical Preparation

The study was approved by the Animal Care and Use Committee of the Wake Forest University School of Medicine (Winston Salem, NC). Male Sprague-Dawley rats (250–300 g) obtained from Harlan (Indianapolis, IN) were used in all experiments. Animals were housed under a 12-h light-dark cycle, with food and water *ad libitum*. For intrathecal drug administration, sterilized 32-gauge tubing (RecathCo, Allison Park, PA) connected

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to 8.5-cm Tygon[®] external tubing (Saint-Gobain Performance Plastics, Akron, OH) was inserted during halothane anesthesia, as previously described.¹⁰ The catheter was passed caudally from the cisterna magnum to the level of lumbar enlargement (7.5–8.0 cm). Only animals without evidence of neurologic dysfunction after catheter insertion were studied. Correct position of the lumbar catheter was verified in a subset ($n = 68$) of animals after surgery. All studies were performed at least 7 days after insertion of the intrathecal catheter. Paw incision was performed as described by Brennan *et al.*¹¹ Animals were anesthetized with halothane; the plantar surface of the left hind paw was prepared with 70% ethanol; and a 1-cm longitudinal incision was made through the skin and fascia, starting 0.5 cm from the edge of the heel and extending toward the toes. The plantaris muscle was elevated and incised longitudinally. The wound was closed with two silk 5.0 sutures.

Behavioral Testing

For determining withdrawal threshold, rats were placed individually in plastic cages with a plastic mesh floor. Animals were tested after acclimation to the environment, typically 30 min after being placed in the cage. Withdrawal threshold to punctuate mechanical testing was determined using calibrated von Frey filaments (Stoelting, Wood Dale, IL), beginning with the 2.0-gauge filament. Filaments were applied vertically to an area adjacent to the wound at the heel for 4 s while the hair was bent. Brisk withdrawal or paw flinching was considered a positive response. In the absence of a response, the filament of next greater force was applied. In the presence of a response, the filament of next lower force was applied. The tactile stimulus producing a 50% likelihood of withdrawal was determined using the up-down method, as described by Chaplan *et al.*¹² Tests were performed in duplicate, with an approximate 3-min test-free period between withdrawal responses, and their average was used. Studies were performed on the first day after paw incision surgery.

Experimental Treatments and Drugs and Their Administration

All drugs experimental treatment was carried out 24 h after plantar incision.

The α_2 -adrenoceptor agonists used in this study were clonidine hydrochloride (non α_2 -adrenoceptor subtype selective, molecular weight of 267 g/mol, nonsubtype selective; Sigma Chemical, St. Louis, MO) and ST91 hydrochloride (α_2 non-A preferring, molecular weight of 254 g/mol; Boehringer Ingelheim, Ridgefield, CT). Animals received cumulative dosing at 40-min intervals of intrathecal clonidine (19, 56, 190 nmol) or at 60-min intervals of intrathecal ST91 (20, 59, 197 nmol). Timing of cumulative injections and dose range were determined in pilot experiments with each drug. Dose-

response curves were constructed after conversion of withdrawal thresholds to percent maximum possible effect. Percent maximum possible effect was defined as: $100 \times (\text{postdrug response} - \text{baseline}) / (\text{pre-paw incision threshold} - \text{baseline})$. Agonists were administered intrathecally in volumes of 10 μl , and thresholds for withdrawal were determined 40 min after clonidine administration and 60 min after ST91 injection.

The α_2 -adrenoceptor subtype antagonists were BRL44408, selective for the α_2 -A-adrenoceptor subtype, and ARC239, selective for α_2 -non-A-adrenoceptor subtypes (both from Tocris Cockson, Ballwin, MO). Antagonists or vehicles were injected intrathecally in a volume of 10 μl 15 min prior to agonist administration. Based on pilot experiments, we used probe doses of 94 nmol clonidine (25 μg) and 59 nmol ST91 (15 μg), and doses of 9.4 or 94 nmol BRL ($n = 7$ –10 animals) and of 9.4 or 94 nmol ARC ($n = 5$ –9 animals). All studies were conducted with the investigator blinded to antagonist or vehicle administered. Drugs were dissolved in normal saline or, when necessary, in 2-hydroxypropyl- β -cyclodextrin (Sigma Chemical).

Muscarinic and nicotinic cholinergic receptor antagonism was performed with intrathecal injection of atropine sulfate (44 nmol) and mecamylamine hydrochloride (491 nmol). We have previously shown that the intrathecal doses of selected antagonists inhibit corresponding cholinergic and α_2 -adrenoceptor ligands in rats subjected to mechanical stimuli.^{8,13}

Statistical Analysis

Data are represented as mean \pm SD. Dose-dependence of clonidine and ST91 was evaluated by linear regression analysis. Paw withdrawal thresholds in response to mechanical stimulation before and after paw incision were compared using a paired Student *t* test. Effects of individual drugs on withdrawal thresholds were determined using a two-way analysis of variance for repeated measures followed by the Bonferroni correction for appropriate multiple comparisons. $P < 0.05$ was considered significant.

Results

One hundred eighty-nine rats were included in the study. The average withdrawal threshold to punctuate mechanical stimulus before paw incision surgery was 40 ± 14 g and decreased to 4 ± 3 g within 24 h after plantar incision ($P < 0.05$).

Antihypersensitivity Effect of Clonidine and ST91 in Rats after Plantar Incision

ST91 produced antihypersensitivity to von Frey filaments with similar potency but significantly greater efficacy than clonidine (fig. 1). ST91 produced transient

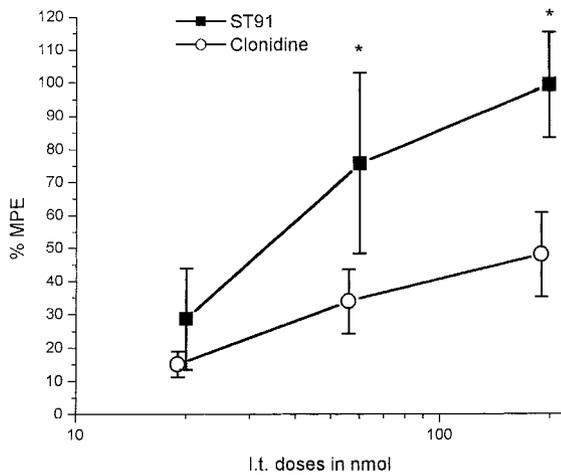


Fig. 1. Dose–response curves for the effects of intrathecally administered clonidine and ST91 on withdrawal threshold to mechanical stimulation near the paw incision site 24 h after surgery. The percent maximum possible effect (%MPE) is the percent return to presurgery withdrawal threshold. Each symbol represents the mean \pm SD of 9 to 10 animals. * $P < 0.05$ versus clonidine.

serpentine tail movements, which were no longer present when withdrawal thresholds were evaluated. Larger doses of clonidine could not be studied due to intense behavioral sedation.

α_2 -Adrenoceptor Antagonism of Clonidine and ST91 in Rats after Plantar Incision

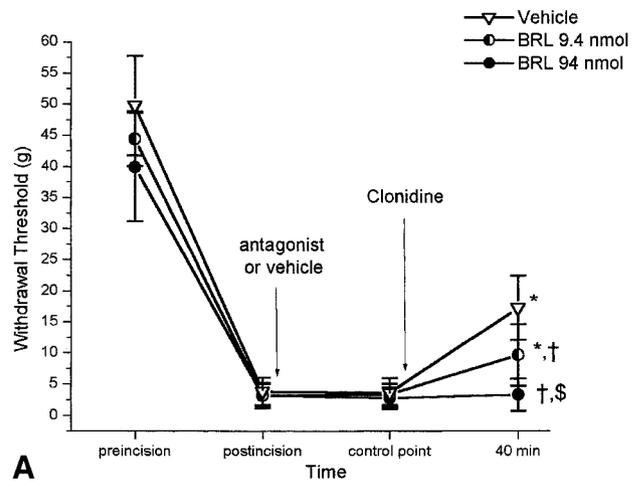
Intrathecal injection of 94 nmol of clonidine increased the withdrawal threshold significantly 40 min after injection (figs. 2A and 2B). Intrathecal injection of BRL44408 and ARC239 significantly inhibited the antihypersensitivity effect of intrathecal clonidine in a dose-dependent manner (figs. 2A and 2B).

Intrathecal injection of 59 nmol ST91 significantly increased the withdrawal threshold 60 min after injection (fig. 3A and B). Intrathecal injection of ARC239 inhibited the antihypersensitivity effect of intrathecal ST91 in a dose-dependent manner (fig. 3A). In contrast, intrathecal injection of BRL44408 failed to influence the antihypersensitivity effect of ST91 (fig. 3B). Intrathecal injection of vehicle or either antagonist alone did not alter the withdrawal threshold (control time points: figs. 2 and 3).

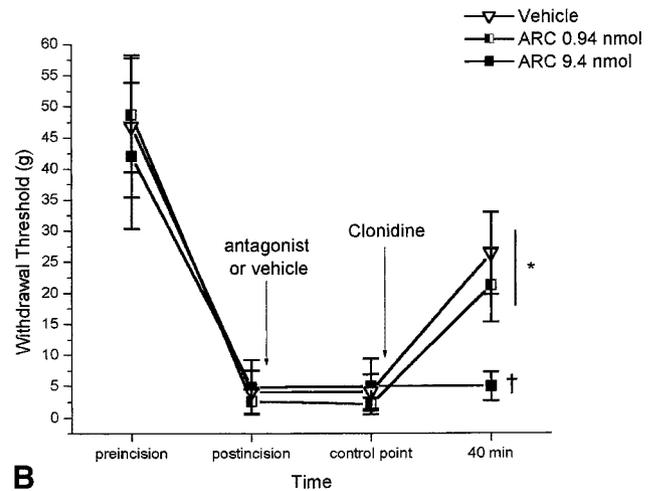
Cholinergic Receptor Antagonist Effects of Clonidine and ST91 in Rats after Plantar Incision

Intrathecal injection of 94 nmol of clonidine increased the withdrawal threshold significantly 40 min after injection (fig. 4A). Intrathecal injection of atropine sulfate and mecamylamine significantly inhibited the antihypersensitivity effect of intrathecal clonidine (fig. 4A).

Intrathecal injection of 59 nmol ST91 increased the withdrawal threshold significantly 60 min after injection (fig. 4B). Neither intrathecal injection of atropine nor of mecamylamine altered the antihypersensitivity effect of



A



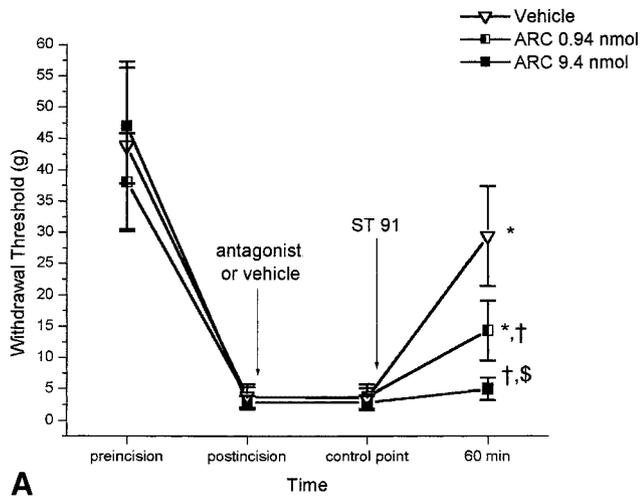
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Fig. 2. Effect of intrathecal pretreatment with vehicle BRL44408 (α_2 A-subtype–preferring antagonist) (A, top) and ARC239 (α_2 non-A subtype preferring antagonist) (B, bottom) on withdrawal thresholds before and after paw incision and after intrathecal injection of 94 nmol clonidine. Each symbol represents the mean \pm SD of 8–10 animals. * $P < 0.05$ versus preincision and control times; † $P < 0.05$ versus vehicle; § $P < 0.05$ versus 9.4 nmol dose of BRL.

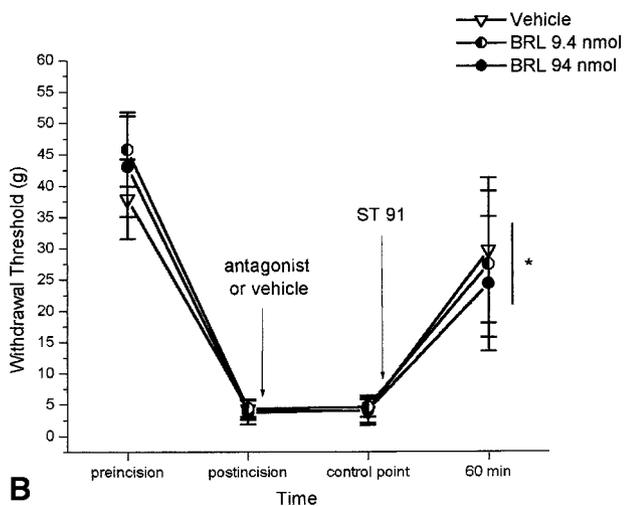
intrathecal ST91 (fig. 4B). Intrathecal injection of vehicle or either antagonist alone failed to alter the withdrawal threshold (control time points: fig. 4).

Discussion

Anesthesiologists deal most commonly with acute pain associated with surgery. Despite the continuing evidence that most patients after surgery experience episodes of severe pain,¹⁴ which can lead to development of chronic pain,¹⁵ most pain research focuses on chronic inflammatory, metabolic, or neuropathic pain conditions. Seminal work by Brennan *et al.* describing the behavioral,¹¹ neurophysiologic,¹⁶ and pharmacologic¹⁷ consequences and properties of surgery clearly indicate



A



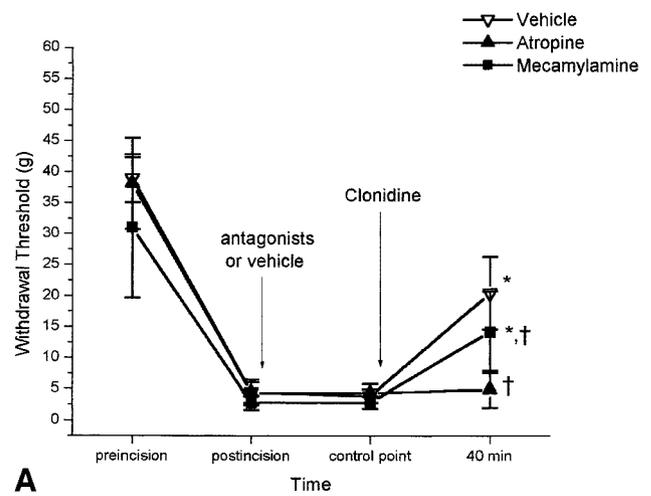
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Fig. 3. Effect of intrathecal pretreatment with vehicle ARC239 (α_2 non-A-subtype-preferring antagonist) (A, top) and BRL44408 (α_2 A-subtype-preferring antagonist) (B, bottom) on withdrawal thresholds before and after paw incision and after intrathecal injection of 59 nmol ST91. Each symbol represents the mean \pm SD of 5–9 animals. * $P < 0.05$ versus preincision and control times; † $P < 0.05$ versus vehicle; \$ $P < 0.05$ versus 0.94 nmol ARC.

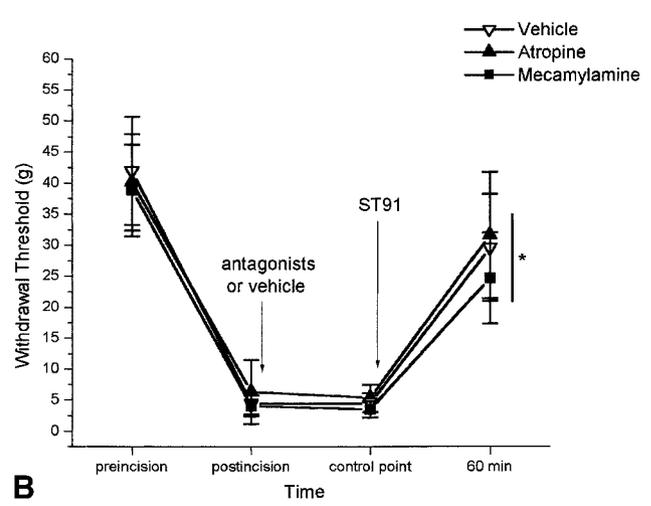
that postoperative pain differs in many fundamental ways from these other conditions, and we and others have argued that the relative lack of basic research in this area hampers better treatment for our postoperative patients.¹⁸ The current study uses the method developed by Brennan *et al.*^{11,16,17} and carries important implications for drug development and drug mechanisms.

Antinociceptive Potency of Intrathecal Clonidine Varies across Pain States

Like opioids, cyclooxygenase inhibitors, and glutamate receptor antagonists, the analgesic potency and maximum efficacy of intrathecal or epidural clonidine depends heavily on the type of clinical pain it is used to treat. This was first noted when the bolus or continuous infusion doses of epidural or intrathecal clonidine to



A



B

Fig. 4. Effect of intrathecal pretreatment with vehicle, atropine (a muscarinic cholinergic receptor antagonist), and mecamylamine (a nicotinic cholinergic receptor antagonist) on withdrawal thresholds before and after paw incision and after intrathecal injection of 94 nmol clonidine (A, top) and of 59 nmol ST91 (B, bottom). Each symbol represents the mean \pm SD of 7–10 animals. * $P < 0.05$ versus preincision and control times; † $P < 0.05$ versus vehicle.

effectively treat neuropathic pain¹⁹ were found to be less than 25% those needed to treat postoperative^{20–22} or obstetric pain.²³ Similarly, potency of clonidine to reduce experimentally induced allodynia was increased compared to treatment of acute noxious stimuli in normal volunteers,²⁴ indicating a shift in drug action or mechanism in the presence of central sensitization. These clinical observations are paralleled in studies with rodents, where generation of hypersensitivity from nerve injury increases potency and efficacy of intrathecal clonidine.^{25,26} The current study in rats suggests that hypersensitivity to mechanical stimuli surrounding a skin incision lies closer in this regard to the normal condition than that following nerve injury. Intrathecal clonidine's potency in the current study of postoperative hypersensitivity is similar to that observed in rats to acute noxious stimuli.²⁷

α_2 -Adrenoceptor Pharmacology of Intrathecal Clonidine Varies across Pain States

All three α_2 -adrenoceptor subtypes (A, B, and C) are present in rat spinal cord,²⁸ with a preponderance of α_2 A and α_2 C subtypes.²⁹ Anatomic and pharmacologic evidence suggests a shift in analgesic mechanisms of intrathecal clonidine from α_2 A predominant in the normal rat to α_2 C predominant after nerve injury. In the normal rat, antinociception of clonidine is reduced by acute knockdown of α_2 A adrenoceptors using antisense oligodeoxynucleotides³⁰ and reversed by the α_2 A adrenoceptor preferring antagonist, BRL44408.⁸ Following nerve injury there is a loss of α_2 A adrenoceptor immunoreactivity in the spinal cord, but no change or an increase in α_2 C adrenoceptor immunoreactivity,³¹ and antinociception of clonidine in this case is unresponsive to antagonism by BRL44408, but reversed by the α_2 non-A-adrenoceptor antagonist, ARC239.⁸

The current study suggests that the mix of α_2 -adrenoceptor subtypes responsible for clonidine antinociception following surgery is intermediate between that in the normal animal and that in the neuropathic animal. Thus, BRL44408 reversed clonidine's effect in the current study, indicating a role for α_2 A adrenoceptors, unlike the results after nerve injury. ARC239 was similarly potent to BRL44408 to reverse clonidine's effect in normal animals,⁸ but was nearly 10-fold more potent to reverse clonidine's effect after surgery in the current study, indicating a partial, but not complete shift to an α_2 non-A-adrenoceptor mechanism.

Reliance of Intrathecal Clonidine on Spinal Cholinergic Systems Varies across Pain States

Stimulation of spinal cholinergic systems by intrathecal injection of α_2 -adrenoceptor agonists is widely documented in humans and animals. Thus, intrathecal clonidine increases acetylcholine concentrations in cerebrospinal fluid³² and analgesic effect of clonidine is potentiated by intrathecal neostigmine in humans.³³ The reliance of antinociception from clonidine on this spinal cholinergic interaction varies between normal and nerve-injured animals. Thus, intrathecal clonidine antinociception to acute noxious thermal stimuli in normal rats is unaffected by intrathecal atropine or mecamlamine (unpublished observations, Xavier Paqueron, M.D., Research Fellow, Department of Anesthesiology, Wake Forest University School of Medicine, June 2000), but the reversal of hypersensitivity by clonidine following spinal nerve ligation is completely blocked by intrathecal atropine and partially blocked by intrathecal mecamlamine.¹³ The current study suggests that postoperative hypersensitivity most resembles nerve injury-induced hypersensitivity in this regard since the effect of clonidine was completely antagonized by atropine and partially by mecamlamine. Should these data apply to humans, they provide a rationale for the study of epi-

dural clonidine-neostigmine combinations for postoperative analgesia.

ST91 Antinociception Reflects Different Pharmacology from Clonidine

ST91, the diethyl derivative of clonidine, was described more than 25 yr ago to explain the lack of hypotensive and sedative properties of clonidine when administered systemically, originally ascribed to its hydrophilic nature.³⁴ Shortly thereafter, it was demonstrated to produce antinociception after intrathecal injection in rats,³⁵ but with a different structure activity relation for blockade by α_2 -adrenoceptor antagonists than clonidine or dexmedetomidine,^{6,36} and with differential cross-tolerance with these agents,³⁷ suggesting it acted on different α_2 -adrenoceptor subtypes. More recent observations of synergistic interactions between dexmedetomidine and ST91 reinforce these early studies.⁷ As in normal animals and those with spinal nerve ligation-induced hypersensitivity,⁸ the effect of ST91 after surgery in the current study was reversed by the α_2 non-A-adrenoceptor antagonist ARC239 but was unaffected by the α_2 A-adrenoceptor-preferring antagonist BRL44408 or the cholinergic antagonists.

ST91 could represent an improvement on clonidine in the treatment of postoperative pain. Although ST91 exhibits less intrinsic activity than clonidine or dexmedetomidine in normal rats,³⁸ it is more effective than dexmedetomidine for antinociception in some strains of rats and in some thermal assays.⁷ ST91 was more efficacious than clonidine in the current study, although whether this was due to a partial agonist effect of clonidine could not be determined, due to dose-limiting sedation by this agent. Sedation and hypotension limit the use of clonidine in the treatment of postoperative pain.³⁹ ST91 does not cause sedation after systemic or intrathecal administration, nor does it reduce blood pressure after intrathecal administration in several species.^{5,34,35,40} Of course, human trials must await proper preclinical chemistry, formulation, stability, toxicity, and neurotoxicity studies.

In summary, intrathecal clonidine partially reverses the reduced withdrawal threshold to mechanical stimulation near a paw incision in rats through the actions of α_2 A- and α_2 non-A-adrenoceptor subtypes. This is also a mechanism that involves stimulation of spinal cholinergic receptors. These data suggest that the proportion of α_2 -adrenoceptor subtypes activated by clonidine to produce antinociception in the postoperative state differs from the normal animal with acute noxious stimuli and the nerve-injured animal with hypersensitivity. ST91 was more efficacious than clonidine, perhaps because larger relative doses could be administered without sedation, and it relied entirely on α_2 non-A-adrenoceptor subtypes. These data add to the growing literature indicating that postoperative pain exhibits a unique pharma-

colony of analgesia compared with acute experimental or chronic nerve injury pain. The data also provide the rationale for the clinical study of epidural clonidine-neostigmine combinations and for development of ST91 for clinical trials.

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